AD_____

Award Number: W81XWH-07-1-0626

TITLE: The Influence of Neuronal Activity on Breast Tumor Metastasis to the Brain

PRINCIPAL INVESTIGATOR: Dr. Anna Majewska
Edward B. Brown, Ph.D.

CONTRACTING ORGANIZATION: University of Rochester
Rochester, NY 14642

REPORT DATE: September 2008

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	**2. REPORT TYPE**	**3. DATES COVERED** *(From - To)*
01-09-2008	Annual	13 Aug 2007-12 Aug 2008

4. TITLE AND SUBTITLE
The Influence of Neuronal Activity on Breast Tumor Metastasis to the Brain

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-07-1-0626

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Dr. Anna K. Majewska
Edward B. Brown, Ph.D.

E-Mail:

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of Rochester
Rochester, NY 14642

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT
In this grant we aim to use our insights gained in the study of developmental neuroscience to affect breast tumor metastasis to the brain. Specifically, we are focusing on our finding that synaptic plasticity is dependent on the remodeling of the extracellular matrix and that this remodeling is elicited by the altered activity of neurons. Because breast tumor metastasis is sensitive to the extracellular environment we believe that by altering neuronal activity we could also affect breast tumor metastasis to the brain. Significant progress during this reporting period includes the establishment and characterization of a model of breast tumor metastasis to the brain in the mouse and the testing of a neurological stimulant in curtailing breast tumor brain metastasis. Our preliminary data suggests that stimulants may affect the ability of circulating cells to enter and grow within the central nervous system.

15. SUBJECT TERMS
brain, extracellular matrix, stimulant, depressant, neuronal activity, diffusion

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON**
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**			USAMRMC
U	U	U	UU	9	**19b. TELEPHONE NUMBER** *(include area code)*

Table of Contents

	Page
Introduction……..	**1**
Body……..	**1**
Key Research Accomplishments……..	**5**
Reportable Outcomes……	**5**
Conclusion……	**5**
References……	**6**
Appendices……	

Introduction

Most anticancer therapies rely on long standing notions of controlling cell division. There have been few, if any, completely new approaches toward the control of tumors in general and breast cancer in particular. In contrast, we propose to exploit the knowledge gained from recent and ongoing work in the field of neuroscience to both predict and prevent breast tumor metastasis in the brain. Specifically we expect that normal mechanisms used by neurons to alter the extracellular matrix as a result of changes in neuronal firing also affect the ability of breast tumor cells to enter the brain, move about, and grow into a tumor. This innovative insight allows us to immediately test the antimetastatic ability of three drugs *already approved for use in the clinic* and hence offers the possibility of extremely rapid clinical application of our ideas. This is the primary reason we believe that this proposal has extremely high immediate impact and will greatly advance the treatment of breast cancer. We also hope to that our work will lead to a whole new way of thinking about breast cancer metastasis to the brain, and lead ourselves and others to explore other clinically approved therapies targeting neurons to see if they alter the extracellular matrix and impact metastasis. Lastly, we are performing some important basic biology that we think establishes a whole new field in cancer research, specifically the exploration of the molecular and physical mechanisms of extracellular matrix modification by neurons and its inhibition of metastasis.

Body

In order to carry out the goals of our proposal we first needed to establish a mouse model of breast tumor metastasis to the brain in our laboratory. Our initial proposal was to use the MDA-MB231 human metastatic ductal breast carcinoma cells, a well established model of human breast cancer, injected intracardially in immuno-deficient mice. However, we found that our preliminary experiments using these cells did not yield brain tumors when the cells were injected intracardially. Additionally, these cells did not appear to grow when injected intracranially suggesting that this cell line has difficulty growing within the brain environment and hence is an inappropriate model of breast tumor metastasis to the brain.

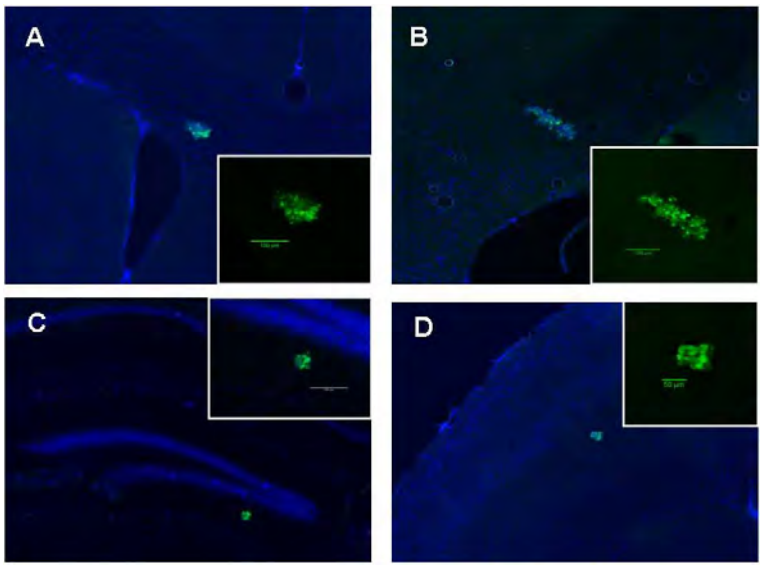

Figure 1. Breast Tumor Metastases to the Brain after Intracardiac Injection

MDA-MB-231BR-YFP cells establish metastases in the Corpus Callosum (A: white matter tumor), thalamus (B), hippocampus (C) and cortex (D). Green is YFP expressed by tumor cells, blue is DAPI nuclear counterstain. Insets are higher magnification images in the YFP channel. Scale bars = 100 μm (A-C); 50 μm (D).

Because of these results we spent the first 6 months of this funding period developing a different mouse model in the lab. We obtained MDA-MB231BR cells (a generous gift from T. Yoneda). This cell line was generated by intracardially injecting MDA-MB231 cells and then extracting brain metastases. This procedure was repeated 6 times to generate a breast tumor cell line that readily metastasizes to the brain[1]. To enable easy scanning of brain sections for small metastases we stably transfected these cells with the fluorescent protein Venus (a version of YFP) in our laboratory before starting our experiments.

In our early experiments with MDA-MB231BR cells we used SCID mice which are immunodeficient and are often used in cancer studies with human cells. However, we found that the variability in the tumor load observed in the brain following intracardiac injections in these mice was very high and would make it difficult to observe meaningful differences in metastasis in drug-treated mice. In 7 mice, we observed an average of 4.7±8 tumors per brain. A visual measure of proper needle position in intracardiac injections is a burst of bright red blood into the hypodermic needle: in SCID mice this burst was almost never visible possibly because it is too difficult to keep these mice lightly anesthetized. We decided to change mouse strain and have been using nude mice which also have a compromised immune system, thus allowing the growth of human tumor cells. In nude mice the burst of blood into the hypodermic needle is robust and readily visible. With these mice we have obtained much a more reproducible metastatic burden in the brain following intracardiac injection. For comparison, in our preliminary study with 7 nude mice we obtained an average of 28±18 tumors per brain.

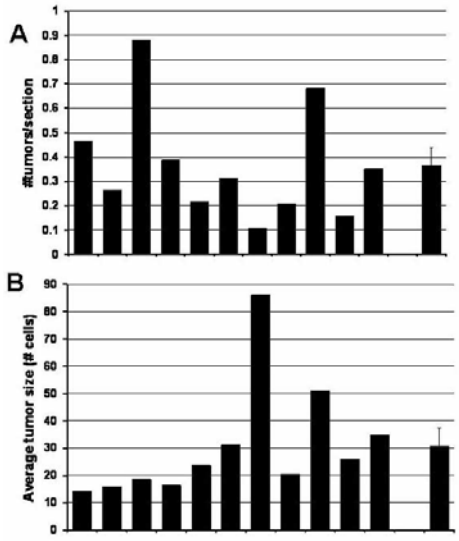

Figure 2. Quantification of tumor metastasis in control animals.

A. Number of tumors/section analyzed in 11 injected animals. The last bar in the graph represents the average and the error bars show the standard error. Notice that all nude mice had detectable tumors.

B. Average tumor size (number of cells counted per tumor) in each individual animal. The last bar represents the group average and standard error.

To further understand characterize this model, we carried out control experiments to characterize the metastasis pattern of MDA-MB231BR cells. Nude mice were intracardially injected with a 100 µl volume containing 200,000 cells. After 3 weeks the animals were deeply anesthetized and perfused with saline followed by fixative. Their brains were removed, dehydrated and sectioned to a thickness of 50 µm. Sections were scanned under epifluorescence and tumors were identified (Figure 1). The number of cells comprising a tumor as well as tumor location within the brain was noted. Brain metastases were identified in all the mice although the number of tumors varied (normalized to the number of sections obtained from individual mouse brains – 73±2 sections were analyzed per mouse). On average 0.37±0.07 tumors/section were identified. The range was 0.11-0.88 tumors/section (Figure 2a). Tumors ranged in size from 3 cells to 270 cells. The average tumor size was 31±7 cells (Figure 2b). The majority (80±3%) of the tumors were found in the gray matter, 18±3% were located at the gray/white matter interface, and

very few tumors (2±1%) were located in the large white matter tracts (Figure 3a). 13±3% of tumors were located in the cerebellum, 42±8% were found in subcortical areas, 41±10% in the cerebral cortex, and 4±4% in the hippocampus (Figure 3b and Figure 1).

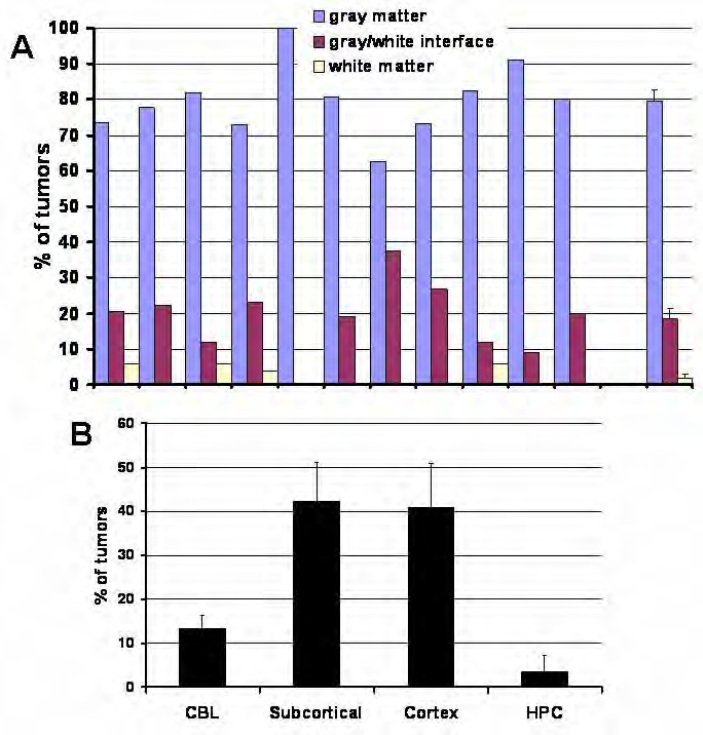

Figure 3. Distribution of tumor metastases within the brain.

A. Percentage of total tumors identified within gray matter, white matter and the gray/white matter interface. The last set of three bars represents the average for the control group. Notice that the majority of tumors are established within the gray matter.

B. Distribution of brain metastases between different brain areas. The majority of tumors were found in cortical and subcortical areas with few being present in the cerebellum (CBL) and hippocampus (HPC).

Aim 1: Drugs known to increase neural activity (and hence reduce spine motility) inhibit breast tumor metastasis to the brain.

After establishing the baseline of tumor growth within the brain after intercardiac injection of breast cancer cells, we turned to exploring whether stimulants which increase neuronal activity affect this metastasis. We proposed to use three different drugs to alter brain activity: caffeine, methylphenidate and modafinil. While the smallest effect may be expected from caffeine which is not as potent as the other drugs, we decided to begin our experiments with caffeine because it can be orally delivered and thus is easier to work with than the other injectable drugs. We pretreated nude mice for 5 days with caffeine by providing the animals with 0.5g/L of caffeine in their drinking water. After 5 days of treatment MDA-MB231BR-YFP cells were injected intracardially and caffeine treatment was continued throughout the 3 week survival period. Interestingly, animals treated with caffeine appeared to have fewer tumors (0.19±0.02 tumors/section (76±3 sections were examined); 7 animals; $p<0.05$ t-test when compared to controls; Figure 4a). The average number of cells per tumor was not statistically significantly different than in control conditions (29±8 cells; $p>0.05$; Figure 4b), suggesting that possibly caffeine affected the ability of breast tumor cells to establish tumors within the brain but not to grow once established. Further supporting the idea that caffeine causes differences in "seeding", caffeine treated animals had a much larger proportion of white matter tumors although the distribution between different brain regions was not significantly different (Figure 5).

We are currently in the process of analyzing data from animals treated with methylphenidate to determine whether the effect of caffeine on breast tumor metastasis is a general feature of stimulants of brain activity.

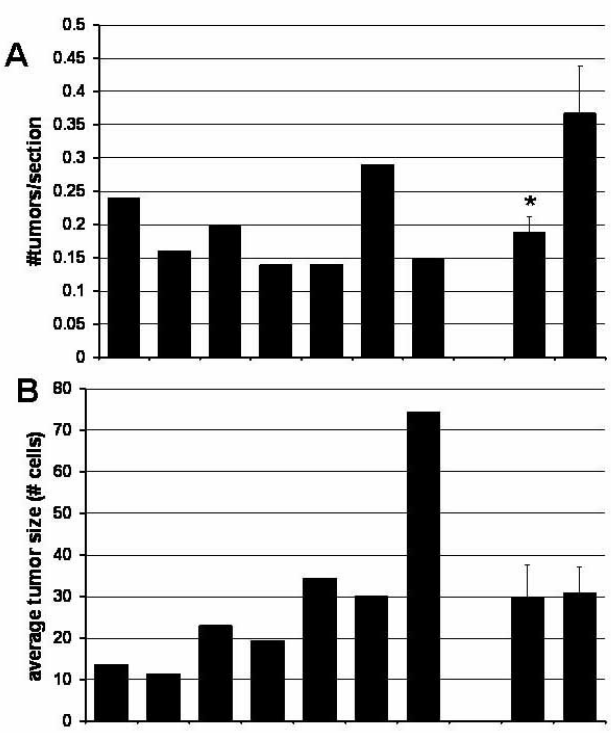

Figure 4. Quantification of tumor metastasis in caffeine-treated animals.

A. Number of tumors/section analyzed in 7 injected animals. The last bars in the graph represent the average number of tumors/section in caffeine-treated (left) and control (right) mice. Notice that caffeine-treated mice have significantly fewer tumors in the brain. * indicates $p<0.05$ t-test.
B. Average tumor size (number of cells counted per tumor) in each individual animal. The last bars in the graph represent the average number of cells/tumor in caffeine-treated (left) and control (right) mice. Notice the lack of difference between tumor size in the two groups.

Error bars show the standard error.

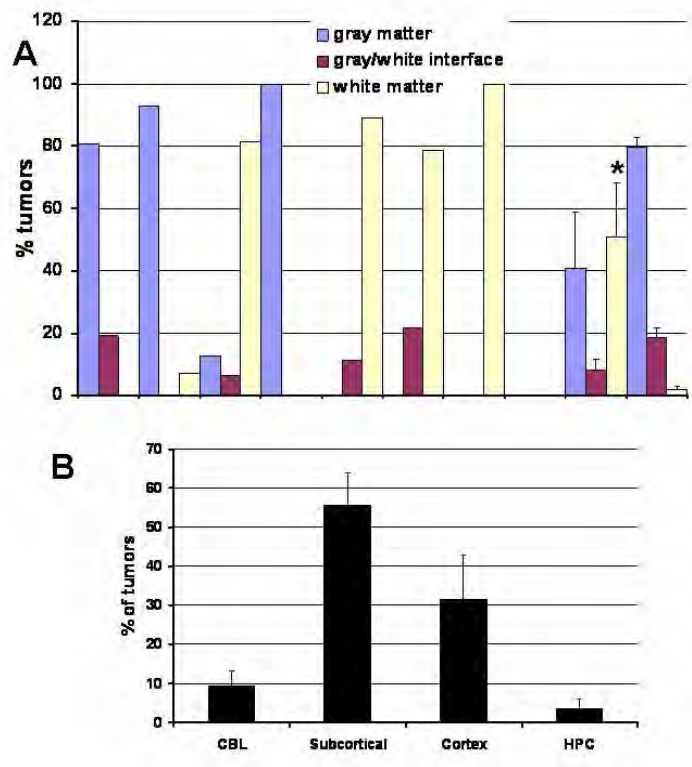

Figure 5. Distribution of tumor metastases within the brain of caffeine-treated animals.

A. Percentage of total tumors identified within gray matter, white matter and the gray/white matter interface. The last two sets of three bars represents the average for the caffeine-treated group (left) and the control group (right). Notice the increase in the proportion of tumors established in the white matter in caffeine-treated animals.
B. Distribution of brain metastases between different brain areas in caffeine-treated animals. The majority of tumors were found in cortical and subcortical areas with few being present in the cerebellum (CBL) and hippocampus (HPC) similarly to control animals.

In summary, while we encountered some unforeseen delays in establishing our mouse model of breast tumor metastasis to the brain, and spent a significant amount of time during this funding period establishing this model, we are currently making good progress toward completing Aim 1. We have finished our analysis of the effects of one of the three drugs we proposed (caffeine), and are in the data analysis stage with another of the drugs (methylphenidate). Experiments with modafinil are planned to start next month. In the last year of the grant we plan on finishing the experiments in Aim 1 and starting experiments to look for tPA activity (Aim 2) and changes in diffusion in treated animals (Aim 3).

Key outcomes

1. We have established and characterized a murine model of breast tumor metastasis in our laboratory
2. We have shown that caffeine treatment alters the rate of breast tumor metastasis to the brain and the location of brain metastases but not their size.

Reportable Outcomes

Presentations:

Zettel. M., Cash, S.S., Brown, E.B., Majewska, A. (2008) Intracardiac injection of MDA-MB-231BR cells in Nu/Nu mice as a model of breast tumor metastasis to the brain. *Soc. Neurosci. Abstr.*

Zettel. M., Cash, S.S., Brown, E.B., Majewska, A. (2008) The influence of neuronal activity on breast tumor metastasis to the brain. *DoD Era of Hope meeting*

Grants applied for:

"Advanced Imaging of Nervous System Plasticity in Development, Health and Disease"
Principal Investigator: Anna Majewska
Agency: Howard Hughes Medical Foundation *Period:* 5/1/09-4/30/15
Amount: $1,500,000 Direct Costs

"Brain Activity and Brain Tumor Infiltration"
Principal Investigator: Anna Majewska *Effort:* 20%
Agency: The Pardee Foundation *Period:* 10/1/08-9/30/09
Amount: $99,921 Direct costs/$124,921 Total costs

Cell lines generated:

MDA-MB231BR-YFP

Conclusion

Our starting hypothesis was that neuronal activity within the brain could affect breast tumor metastasis to this organ. This hypothesis came out of our experiments with synapses (the structures that allow neurons to communicate with one another) that showed that decreasing neuronal activity led to release of tissue plasminogen activator (tPA), degradation of the extracellular matrix and increased synaptic structural changes. We postulated that since breast tumor metastasis is sensitive to the structure of the

extracellular matrix such changes (along with the possible effects of tPA on the blood brain barrier) could affect breast tumor metastasis to the brain. Thus we sought to limit this metastasis by increasing brain activity and limiting the release of tPA. This approach of affecting metastasis indirectly by acting on the host tissue rather than the tumor cells themselves is novel and could provide a radical improvement in the treatment of tumors that target the brain, as the brain is largely protected from conventional chemotherapy but not from drugs which are already approved for use to alter brain activity in neurological diseases. The experiments reported here using caffeine, a known brain stimulant, validate this approach to brain tumor treatment and provide great hope in using this approach in the future. We showed that pre-treating animals with caffeine reduced the brain tumor burden twofold after introduction of human breast tumor cells to the mouse circulatory system. This finding is very exciting and we are in the process of testing other stimulants currently used in the clinic which might provide a more potent reduction of breast tumor metastasis to the brain. Additionally, we are starting our experiments to try to determine the molecular underpinnings of the metastasis reduction, specifically examining the neuronal release of proteases and changes in brain extracellular matrix. These experiments may provide additional targets for therapeutics in the treatment of breast tumor metastasis.

References

1. Yoneda, T., Williams, P.J., Hiraga, T., Niewolna, M., and Nishimura, R., A bone-seeking clone exhibits different biological properties from the MDA-MB-231 parental human breast cancer cells and a brain-seeking clone in vivo and in vitro. *J Bone Miner Res*, 2001. **16**(8): p. 1486-95.